TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED / ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	ATTORNEY'S DOCKET NUMBER P67342US0
	US APPLICATION NO (If known, see 37 CFR 1.5) 09/926603

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/DK00/00281	25 May 2000	25 May 1999

TITLE OF INVENTION

ISOLATION AND CULTURING OF FETAL CELLS

APPLICANT(S) FOR DO/EO/US

John PHILIP -and- Britta CHRISTENSEN

Applicant herein submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ■ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ■ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ■ A proper Demand for Internatl. Preliminary Examination was made by the 19th month from earliest claimed priority date.
5. ■ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ■ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ■ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ■ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ■ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the Internatl. Preliminary Examination report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet compliance with 37 CFR 3.28 and 3.31 is included.
13. ■ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ■ Other items or information:

    International Search Report – EPO
    PCT/IB/304 Form
    First Page of Publication
    International Preliminary Examination Report – with Annexes

US APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
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	CALCULATIONS	PTO USE ONLY
17. ■ The following fees are submitted:		
Basic National Fee (37 CFR 1.492(a)(1)-(5)):		
Internatl. prelim. examination fee paid to USPTO (37 CFR 1.492 (a) (1)) .. $710.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.492 (a) (2)) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) .. $740.00		
Neither international preliminary examination fee (37 CFR 1.492 (a) (3)) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO) **$1040.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.492 (a) (4)) and all claims satisfied provisions of PCT Article 33(2)-(4) $100.00		
Search Report prepared by the EPO or JPO (37 CFR 1.492 (a) (5)) **$890.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of $130.00 for furnishing the **oath or declaration** later than ☐ **20** ■ **30** months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

Claims	**Number Filed**	**Number Extra**	Rate		
Total Claims	**28** - 20 =	-8-	x $18.00	$ 144.00	
Independent Claims	**2** - 3 =	-0-	x $84.00	$	
Multiple Dependent Claim(s) (if applicable)			+ $280.00	$	
TOTAL OF ABOVE CALCULATIONS =				$ 1164.00	
Reduction by 1/2 for filing by **small entity**, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$ 1164.00	
Processing fee of $130 for furnishing the **English translation** later than ☐ **20** ☐ **30** months from the earliest claimed priority date (37 CFR 1.492(f))				$	
TOTAL NATIONAL FEE =				$ 1164.00	
Fee of $40.00 for recording the enclosed **assignment** (37 CFR 1.21(h)). Assignment must be accompanied by appropriate cover sheet (37 CFR 3.28, 3.31).				$	
TOTAL FEES ENCLOSED =				$ 1164.00	
				Amt. to be refunded:	$
				Amt. charged:	$

a. ■ A check in the amount of $ 1164.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. **06-1358** in the amount of $ ____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ■ The Commissioner is hereby authorized to charge my account any additional fees set forth in §1.492 during the pendency of this application, or credit any overpayment to Deposit Account No. **06-1358**. A duplicate copy of this sheet is enclosed.

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JPH&S 3/85

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: John PHILIP et al

Serial No.: New

Filing Date: November 26, 2001

For: ISOLATION AND CULTURING OF FETAL CELLS

PRELIMINARY AMENDMENT

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Prior to initial examination, please amend the above-identified application as follows:

IN THE SPECIFICATION

Please insert the following sentence on line 1, immediately following the title:

--This is a nationalization of PCT/DK00/00281, filed May 25, 2000, and published in English.--

IN THE CLAIMS

Please amend claims 13, 14, 16, 17, 19, 20 and 22 as follows:

13. (amended) The method according to claim 1, wherein the maternal blood sample is diluted before labelling or identification of the fetal cells.

14. (amended) The method according to claim 1, wherein the selective labelling is based on hybridisation of a probe to m-RNA selectively expressed by fetal cells.

16. (amended) The method according to claim 14, wherein the hybridisation probe is directly labelled by having fluorochromes covalently attached thereto.

17. (amended) The method according to claim 1, wherein the selective labelling is based on an antigen-antibody reaction with a protein selectively produced by fetal cells.

19. (amended) The method according to claim 17, wherein the antibody is selected from anti epsilon (ε)antibodies, such as monoclonal unlabelled antibodies, and monoclonal fluorochrome labeled antibodies, anti zeta (ζ) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies,

monoclonal fluorochrome labeled antibodies, and fluorochrome labeled antibodies, anti gamma (γ) antibodies, such as polyclonal (sheep) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies, monoclonal fluorochrome labeled antibodies, and fluorochrome labeled antibodies , anti alpha (α) antibodies and anti beta (β) antibodies.

20. (amended) The method according to claim 1, wherein two or more selective labellings are performed to enhance the probability of identifying the fetal cells in the sample.

22. (amended) The method according to claim 1, wherein the identification of the selectively labelled fetal cells is taking place after spreading the blood sample on a solid surface and detecting labelled cells on the surface.

REMARKS

The foregoing Preliminary Amendment is requested in order to delete the multiple dependent claims and avoid paying the multiple dependent claims fee.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "**VERSION WITH MARKINGS TO SHOW CHANGES MADE.**"

Early action on the merits is respectfully requested.

Respectfully submitted,

JACOBSON HOLMAN PLLC

By _____
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Atty. Docket: P67342US0
Date: November 26, 2001
HBJ:jrc

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE CLAIMS

13. (amended) The method according to claim 1 ~~any of the preceding claims~~, wherein the maternal blood sample is diluted before labelling or identification of the fetal cells.

14. (amended) The method according to claim 1 ~~any of the preceding claims~~, wherein the selective labelling is based on hybridisation of a probe to m-RNA selectively expressed by fetal cells.

16. (amended) The method according to claim 14 ~~or 15~~, wherein the hybridisation probe is directly labelled by having fluorochromes covalently attached thereto.

17. (amended) The method according to claim 1 ~~any of claims 1-13~~, wherein the selective labelling is based on an antigen-antibody reaction with a protein selectively produced by fetal cells.

19. (amended) The method according to claim 17 ~~or 18~~, wherein the antibody is selected from anti epsilon (ε)antibodies, such as monoclonal unlabelled antibodies, and monoclonal fluorochrome labeled antibodies, anti zeta (ζ) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies, monoclonal fluorochrome labeled antibodies, and fluorochrome labeled antibodies, anti gamma (γ) antibodies, such as polyclonal (sheep) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies, monoclonal fluorochrome labeled antibodies, and fluorochrome labeled antibodies , anti alpha (α) antibodies and anti beta (β) antibodies.

20. (amended) The method according to claim 1 ~~any of the preceding claims~~, wherein two or more selective labellings are performed to enhance the probability of identifying the fetal cells in the sample.

22. (amended) The method according to claim 1 ~~any of the preceding claims~~, wherein the identification of the selectively labelled fetal cells is taking place after spreading the blood sample on a solid surface and detecting labelled cells on the surface.

Title

Isolation and culturing of fetal cells.

Introduction

The present invention relates to a method for isolating fetal cells from maternal blood, in particular from maternal blood not having been subjected to concentration or enrichment.

In addition the invention relates to a method for culturing fetal cells.

Background

The examination of fetal cells for early detection of fetal diseases and genetic abnormalities is carried out in connection with many pregnancies, in particular when the maternal age is high (35 years or above) or where genetic diseases are known in the family. Fetal cells may be obtained by amniocentesis, the removal of amniotic fluid from the amniotic cavity within the amniotic sac or by chorion biopsy, where biopsies are taken from the placenta, a so-called invasive sampling.

During pregnancy a variety of cell types of fetal origin cross the placenta and circulate within maternal peripheral blood. The feasability of using fetal cells in the maternal circulation for diagnostic purposes has been hindered by the fact that fetal cells are present in maternal blood in only very limited numbers, reported numbers have been from $1:10^5$ to $1:10^8$ fetal cells per nucleated maternal cells. In addition most fetal cells cannot be distinguished from maternal cells on the basis of morphology alone, but rather must be identified based upon detection of fetal cell markers. However, it would be advantageously to perform fetal diagnosis by a non-invasive procedure, such as a maternal blood sample.

One particular fetal cell type within maternal blood that has been demonstrated to be useful for detecting fetal DNA is the nucleated erythrocyte.

Also, fetal leukocytes have been reported to be present in maternal blood. Leukocytes are one subpopulation of white blood cells found in the blood. There are three types of subsets of leukocytes (which also are referred to as polymorphnuclear leukocytes): neutrophils, basophils and eosinophils. All leukocytes have a distinctive morphology characterized by the nucleus and cellular granules.

Due to the very limited number of fetal cells in maternal blood concentration or enrichment of the maternal blood sample with respect to the fetal cells have been conducted by negative selection, i.e. removal of maternal cells. Enrichment of fetal cells by density gradient centrifugation or by removing maternal cells with an antibody to a cell surface antigen is described in for example US 5,858,649, US 5,731,156, US 5,766,843 and US 5,861,253.

Yet another method of removing maternal cells, in particular maternal erythrocytes, is by lysing, again optionally combined with immunologic methods for removing the maternal cells.

Another selection procedure is positive selection, for example by use of CD71 antibodies.

US 5,861,253 describes enrichment either before and/or after labelling of the fetal cells for further analysis.

It is however, a problem that due to the enrichment procedures some of the fetal cells may also be removed leading to even fewer fetal cells in the blood sample to be analysed.

In order to increase the number of fetal cells attempts of culturing the cells have been carried out in the prior art. There are a few publications describing successful methods for culturing fetal cells from peripheral blood of pregnant women. Lo et al, (Lancet 1994, 344, 264) cultured cells from five pregnant women carrying male fetuses. In two cases they examined samples before culturing with negative results. In all cases they ascertained cells by Fluorescence in situ hybridization (FISH) after culture. Very few details of the culture methodology are given, and the results have not been repeatable with the available information. In a study by Little et al, (Blood,

273, R 1829) culture was used as part of their isolation method and male cells were found after various sorting/enrichment procedures. Also Jansen et al, (Prenatal Diagnosis, 1999, 19, 323) developed a method for culturing cord blood cells in a model system for isolation of fetal nucleated red blood cells. However, none of the methods have shown a significant increase in fetal cells after culture.

Summary of the invention

It is an object of the present invention to provide for a method for isolating fetal cells from maternal blood, wherein the blood sample has not been substantially enriched, such as by performing, on a sample of maternal blood from which at the most 50% of the anucleated maternal cells thereof and/or at the most 50% of the nucleated maternal cells thereof have been removed, selective labelling of fetal cells in the maternal blood sample, identifying the selectively labelled fetal cells, and specifically isolating substantially only the selectively labelled fetal cells.

Thus, according to the invention a method is obtained whereby the risk of removing fetal cells from the maternal blood sample before analysing the sample has been greatly reduced.

Detailed description of the invention

The present invention reveals a novel method of isolating fetal cells from maternal blood. The present method has proven to be optimised in relation to isolating methods described in the prior art, and the present invention represents a method having beneficial properties technically and financially. In the light of the naturally occurring ratio between maternal cells and fetal cells the present invention presents a method wherein fetal cells are isolated from maternal blood without prior enrichment or concentration of the sample, providing for a method of isolation by which the risk of losing fetal cells due to enrichment or concentration procedure has been reduced.

A major difference between maternal and fetal red blood cells is the latter having a nucleus, i.e. maternal red blood cells are anucleated. Furthermore, maternal blood contains three types of nucleated fetal cells, nucleated erythrocytes, syncytiotrophoblasts and leukocytes. It is an object of the present invention to provide for a method

wherein the blood sample from which the fetal cells are isolated is substantially not enriched or concentrated prior to isolation by removing any of the maternal cells.

Accordingly, in order to reduce the risk of removing fetal cells in the preparation of maternal blood samples to be analysed it is an object of the present invention that at most 50 % of the maternal cells of the maternal blood sample have been removed or will be removed before or after the labelling of the fetal cells, thus that substantially no enrichment of the sample is carried out before identification of the cells. In a more preferred embodiment at most 20 % of the maternal cells have been removed, such as at the most 15% of the maternal cells, such as at most 10 % of the maternal cells, more preferred at most 5 % of the maternal cells, more preferably at most 2.5 % of the maternal cells, most preferred at most 1 % of the maternal cells.

According to the invention it is even more preferred that substantially none of the maternal cells have been removed from the sample.

Thus, it is encompassed by the present invention that at most 20 % of the maternal nucleated blood cells have been or will be removed and/or at most 20 % of the anucleated red blood cells have been or will be removed.

Even more preferred is a method wherein substantially none of the nucleated blood cells or anucleated red blood cells have been removed from the sample. Thereby the sample may be used as such directly after taking the maternal blood sample.

It is desirable to obtain as large a maternal blood sample as possible in order to increase the total number of fetal cells. However, due to proctical problems the sample must be within certain limits. Accordingly, the size of the maternal blood sample is preferably in the range of 0,5 to 40 ml, such as in the range of 5 to 40 ml, such as from 10 to 30 ml.

Also, according to the invention it is preferred to dilute the sample before labelling or before identification of the fetal cells (to facilitate the identification of the fetal cells). The sample may be diluted at least 1.5 times, such as twice, more preferred at least three times, such as five times by adding isotonic buffers, such as saline solutions,

phosphate buffered saline solutions, PBS, and/or suitable growth media, such as basal media, and tissues growth media.

The selective labelling of the fetal cells may be carried out by any suitable method. Fetal cells may be distinguished from maternal cells by the specific recognition of a fetal cell antigen or they may be distinguished by staining with a labelled antibody to a protein selectively produced by fetal cells or they may be distinguished from maternal cells by the specific recognition of DNA or RNA encoding a protein selectively or substantially selectively produced by fetal cells.

Accordingly, it is an object of the present invention to provide for the selective labelling of fetal blood cells in the maternal blood sample based on hybridisation of a probe to m-RNA selectively expressed by fetal cells.

Preferably, fetal-cell-specific RNA sequences are used as fetal cell markers. Such RNA is generally messenger RNA (mRNA). The presence of such RNA indicates that the gene for the fetal protein is being transcribed and expressed. (The probes used to identify fetal cells in a sample containing fetal and maternal cells include nucleic acid molecules, which comprise the nucleotide sequence complementary to the nucleotide sequence of the RNA molecule encoding a specific protein. Fetal cells contain distinct mRNAs or RNA species that do not occur in other cell types. The detection of these RNAs, whether as mRNA can serve to identify cells, or even subcellular fractions of cells fetal or embryonic in origin). According to the present invention the m-RNA to be detected may be coding for a protein selected from the group consisting of embryonic hemoglobin, such as ε and zeta globin chains, and fetal hemoglobin, such as gamma and alpha globin chains.

Further, according to the present invention DNA probes (oligos) for the hybridisation are directed against embryonic cell RNA, such as for ε and zeta globin chains, and for fetal hemoglobin, such as for gamma and alpha globin chains. A DNA probe may be synthesised as an oligodeoxynucleotide using a commercial synthesiser. Probes may be comprised of the natural nucleotide bases or known analogues of the natural nucleotide bases.

Yet further to the invention the hybridisation probe for the DNA or RNA discussed above is selected from peptide nucleic acid (PNA) probes and other synthetic molecules capable of Watson Crick-base pairing with the fetal m-RNA.

In one embodiment of the invention the probe as discussed above, such as a synthetic DNA probe, is directly labelled, by having fluorochromes covalently attached thereto. The binding of such probes to the cell may be observed under a microscope as a bright fluorescence or may be detected by a fluorimetric apparatus.

Instead of direct labelling or in addition to the direct labelling in another embodiment the probes are indirectly labelled with biotin or enzymes for example, such as alkaline phosphatase.

By using a combination of labelling methods it is possible to enhance the signals from the fetal cells, thereby facilitating the identification thereof.

Certain RNA populations are present in high abundance and other fetal or embryonic-specific RNAs are present in low abundance. Several RNA species occur simultanously in fetal cells as opposed to maternal cells. This provides for yet another method of enhancing the distinction between fetal cells and non-fetal cells by the detection of multiple RNA species. Two or more RNA species may be detected using one or more probes for a first RNA sequence and one or more probes for a second RNA sequence. The probes for the first sequence are labelled to provide a first signal, such as a greenish fluorescence, and the probes for the second sequence are labelled to provide a signal that is different from the first signal, such as a reddish fluorescence. When the combination of both signals are detected in a single cell, which in this case would be an orange fluorescence, then both RNAs are found and thus a fetal cell has been detected.

According to another method of the invention the selective labelling is based on an antigen-antibody reaction with a protein selectively produced by fetal cells. Such a protein may be selected from the group consisting of embryonic hemoglobin, such as ε and zeta globin chains, and fetal hemoglobin, such as gamma and alpha globin chains.

In particular the labelling may be carried out by the use of an antibody selected from antibodies against various types of normal globin chains in human hemoglobin, for example anti epsilon (ε) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies, monoclonal biotin and fluorochrome labelled antibodies, and monoclonal fluorochrome labelled antibodies, anti zeta (ζ) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies, monoclonal fluorochrome labeled antibodies, and polyclonal fluorochrome labeled antibodies, anti gamma (γ) antibodies, such as polyclonal (sheep) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies, monoclonal fluorochrome labeled antibodies, and polyclonal fluorochrome labeled antibodies, anti alpha (α) antibodies, and anti beta (β) antibodies.

The fluorochrome is selected to be excited in the wave-length area of the detection means, and furthermore in suitable combination with an optional second labelling. In particular the fluorochromes may be selected from FITC (fluorescein-isofluocyanate) or TRITC (Rhodanine Tetramethyl- isofluocyanate) having excitation at 495 nm and 555 nm, respectively.

In a preferred embodiment of the present invention the labelling is carried out using anti epsilon (ε) monoclonal antibodies or anti zeta (ζ) monoclonal antibodies, more preferably anti epsilon (ε) unlabelled monoclonal antibodies or anti zeta (ζ) unlabelled monoclonal antibodies, or anti epsilon (ε) monoclonal biotin labelled antibodies or anti zeta (ζ) monoclonal biotin labelled antibodies.

The unlabelled antibodies are used as known in the art, by using a second labelling step with eg. second antibodies against the unlabelled antibody, said antibody being labelled as discussed above, such as fluorochrome labelled. By this two-step it may be possible to enhance the signals from the fetal cells.

In another preferred embodiment of the invention the labelling is performed using anti gamma (γ) fluorochrome labeled antibodies, such as FITC labelled antibodies.

In order to enhance the probability and selectivity of identifying the fetal cells or the background of maternal cells by the labelling two or more selective labellings may be performed. The combination of two or more labellings may be a combination of

any of the labellings used for single labelling as well. Accordingly, the combined labelling may be carried out by the use of two or more different hybridisation probes, such as a combination of a DNA probe and a PNA probe for hybridisation with the same fetal RNA or more preferred with different RNAs. Also, two or more different DNA probes (or PNA probes) may be used for hybridisation with different fetal RNAs.

The enhanced selective labelling may also by carried out by the use of two or more antibodies directed against the same protein or different proteins. In this embodiment the labelling with two or more labels may be carried out simultaneously.

In another embodiment a combination of an immunological labelling and a hybridisation labelling may be employed according to the present invention. In this embodiment the labelling is normally carried out sequentially by a first immunological labelling step, then identification of the labelled cells, and then a second step of hybridisation labelling for verification of the identification of the cells labelled by the first step. It is preferred in the first step to use antibodies against gamma globin and in the second step to use hybridisation for epsilon and/or zeta globin and/or gamma mRNA to verify the fetal cells identified.

In yet another embodiment the verification may be conducted by a second step of staining the cell nucleus since the maternal cells probable to be detected will be anucleated maternal cells, i.e. maternal erythrocytes. Thus by verifying that identified cells contain a nucleus it is ensured that fetal cells have been identified. In particular the nucleus staining is selected from 4,6-diamidino-2-phenylindole (DAPI) or propidium-iodide (PI).

An important feature of the present invention is the identification of the labelled fetal cells without the sample being enriched or concentrated with respect to the fetal cells in order to avoid loss of fetal cells. Accordingly, the method according to the present invention comprises identification of the selectively labelled fetal cells.

In one embodiment the identification is performed by spreading the blood sample on a solid surface and detecting the labelled cells on the surface. The detection may be carried out by any suitable means in accordance with the labelling method in ques-

tion. The choice of solid support surface may depend upon the procedure for visualisation of the cells. Some materials are not uniform and therefore shrinking and swelling during in situ hybridisation procedures will not be uniform leading to inaccuracy in the identification procedure. Other autofluoresce support materials will interfere with the determination of low level fluorescence. Support materials according to the invention preferably comprise glass, nylon, nitrocellulose and Scotch tape, and any suitable membranes, such as filtermembranes. Preferably, the collected samples are spread on a support surface in a monolayer for the cells not to overlap one another. Also, the in situ hybridization process according to the invention may carried out on fetal cells attached to a solid support.

Various antibodies have been used to discriminate between maternal and fetal cells as discussed above. In one embodiment the antibodies may be coupled to numerous solid surfaces or supports/substrates, such as containers, columns, wells, beads, or particles by physical or chemical bonding. Alternatively, the antibodies may be coupled to a compound which facilitates the separation step. For example antibodies may be labelled with fluorescent markers and cells to which these labelled antibodies bind may thereby be separated with a cell sorter according to known procedures.

The nucleic acid of the fetal cells can be amplified prior to detection using a known amplification technique, such as the polymerase chain reaction (PCR). Primers for PCR amplification are chosen to specifically amplify a DNA of interest in the fetal DNA.

Due to the large amount of cells to be examined to find the small amount of fetal cells in the blood sample an important factor for the detection equipment is the rate of cells identified per unit of time. For example very fast scanning microscopes may be used for the identification. Also, laser scanners could be used. Preferably the laser scanner is equipped with at least two lasers emitting light with different wavelength capable to excite the various labels on the cells or in one laser emitting several wavelengths.

Preferably, during or after identification of the fetal cells the position of detected labelled cells on the surface is recorded. This provides for the later collection of the detected cells from the position which has been recorded. The position of the detected labelled cells on the supporting surface may be recorded by use of a scanner provided with detectors registering the light emitted from the labelled cells, such photo-multipliers, CCDs, or the like detectors. Thereby it is possible to identify and specifically isolate substantially only the selectively labelled fetal cells.

In a preferred embodiment the scanner is arranged for detecting selectively labelled fetal cells, and when detecting a fetal cell, carrying out a verification step by switching to another wavelength to verify presence of for example staining of the nucleus. It is of importance for the use of the method that a fast scanning system is used, for example a scanner capable of scanning in the range of from 0.1 m/sec to 10 m/sec or faster, such as appr. 1m/sec.

The cells collected according to any of the procedures may be subjected to further identification and/or investigation, such as microscopic and/or molecular identification and/or investigation. The cells may be subjected to investigations of analysing the presence of genetic diseases, for example. The nucleic acid of fetal cells may be analysed for diagnostic or other purposes. For instance the presence or absence of a gene or a gene mutation may indicate the presence of diseases, such as cystic fibrosis. The nucleic acid may additionally be analysed for X or Y specificity. Thus, the presence of a Y chromosome encoded genes or gene products is a qualitative distinguishing feature of the cells of a male fetus.

Verification of the selective identification of fetal cells may be carried out by several methods. In a model system the method may be performed on maternal blood samples from pregnant women carrying a male fetus. The cells isolated may then be analysed for the presence of a Y chromosome, indicative of cells being from the male fetus.

Another verification method, which is usable independent of the sex of the fetus, is verification by use of identification of small tandem repeats (STR) or variable number tandem repeats (VNTR) to detect genetic input from the father, thereby verifying fetal cells, as the only cells in the sample comprising input from the father.

As may be understood from the above the present method may be carried out for the isolation of any kind of rare event cells in a blood sample, and is particular interesting when used for rare event cells being present in very low concentrations, such as those for the fetal cells in maternal blood. This may for example also be true for some cancer forms.

Further, according to the present invention a method for multiplying fetal blood cells, preferably fetal red blood cells, in a cell culture comprising fetal blood cells and other cells, in particular maternal blood cells may be employed. The cultivation may be carried out on any sample comprising fetal blood cells, such as umbilical cord samples from aborted fetuses, or maternal blood samples.

The multiplication of the fetal cells is desirable to achieve due to the use of the present invention. The method according to the invention comprises performing the cell culturing in a culture medium containing growth factors necessary for the multiplication of fetal cells.

The multiplication may be conducted by any suitable method as known to the person skilled in the art.

The method of culturing fetal cells may be combined with the method of selectively isolating fetal cells in any suitable manner. Accordingly, a maternal blood sample may be subjected to cultivation of fetal cells before subjecting the sample to labelling, identification and isolation of the fetal cells. Thereby the starting concentrations of fetal cells in the maternal blood sample are increased without removing any cells originating from the mother. The culture method may also be used in connection with analysis of maternal blood samples that have been pretreated in order to remove some of the maternal cells.

When the labelling and identification procedures allow viable cells to be selected the culture method may be conducted on fetal cells having been identified and isolated according to the isolation method described above, in order to increase the amount of fetal cells for further analysis.

After cultivation the fetal cells may be harvested by any suitable method. In case the colonies obtained are harvested collectively, there will be a very high number of cells of which only some will be from the fetus. Contrary to this, colonies may be harvested individually to increase the rate of fetal cells in the harvested material.

Also, the invention relates to a method of diagnosing a disease in a fetus comprising obtaining a blood sample from the woman pregnant with said fetus, whereby at most 50 % of the maternal nucleated cells have been removed and/or at most 50 % of the maternal anucleated cells have been removed from said blood sample, selectively labelling the fetal cells in the maternal blood sample, identifying the selectively labelled fetal cells, specifically labelling with at least one disease marker the identified fetal cells for diseases, and identifying specifically labelled cells.

The selective labelling and identification of the cells are carried out as described above. The specific labelling with at least one disease marker refers to the labelling of the identified cells with a marker, such as a probe, to a gene or a gene mutation specific for the genetic disease or a chromosome abnormality to be diagnosed.

In a preferred embodiment wherein the fetal cells are isolated before specifically labelling the fetal cells.

Furthermore, the present invention relates to the use of the present method for diagnosing a disease of the fetus, such as a genetic disease. The genetic disease may be any genetic disease or a chromosome abnormality, such as cystic fibrosis, hemophilia, muscular dystrophy, Down' syndrome, Klinefelter, or Turner' syndrome.

The invention is further exemplified by the following non-limiting examples.

Examples

Example 1

Fixation of peripheral venous blood cells before mounting on slides.

The blood is fixed according to table I below and mounted on slides as described.

Table I

Step	Procedure: Fixation and slide preparation.	Time	Tempera-ture
1	100 µl blood. Ad 100 µl Hank's (Ca^{++} and Mg^{++} free) or PBS		R.T.
2	Ad 200 µl 4% paraformaldehyde in PBS (Vortex)	1 hr	37 °C
3	Ad 3 ml PBS (+,- 3%BSA)		R.T.
4	Spin at 1500 rpm	5 min.	R.T.
5	Permeate cells with 1 ml methanol/acetone (1+1, -20°C)(Vortex)	1 hr - o.n*	4 °C
6	Ad 3 ml cold PBS		4°C
7	Spin at 1500 rpm	5 min	R.T.
8	Wash cells in cold PBS (+,- 3%BSA).		4°C.
9	Spin at 1500 rpm	5 min	R.T.
10	Resuspend cells in PBS (aprox. 100 µl) (+,- 3% BSA)		R.T.
11	Prepare slides. Smears on poly-L-lysine coated slides.		R.T.
12	Store slides sealed individually in plasticbags		-80 °C

* o.n. = over night.

The slides prepared are stained with antibodies against globin chains and embryonic globin chains as described below in table II.

Table II

Step	Procedure: Antibody staining of slides	Time	Tempera-ture
1	Wash slides in PBS	2 x 5 min	R.T.
2	Wask slides in 4xSSC	10 min	R.T.
3	Block in 4xSSC/1%BSA/0,5% Boehringer blocking reagent = Buffer A	10 min	R.T.
4	Inkubate with primary (anti-globin chain antibody, 2-3 µg/slide) diluted in buffer A.	30 min humid atm.	R.T.
5	Wash in 4xSSC/0,5% Tween20	2 x 5 min	R.T.
6	Inkubate with biotinylated goat anti-mouse diluted in buffer A.	30 min humid atm.	R.T.
7	Wash in 4xSSC/0,5% Tween 20	2 x 5 min	R.T.
8	Inkubate with fluorochrome conjugated avidin/streptavidin diluted in buffer A	30 min humid atm.	R.T.
9	Wash in 4xSSC/0,5%Tween 20	2 x 5 min	R.T.
10	Wash in 2xSSC	5 min	R.T.
11	Mount in Vectashield with counterstain added (DAPI or PI)		R.T.
12	Store slides in darkness		4 °C

Vectashield is a trademark to Vector Laboratories, USA.

After staining the slides may be stored for later identification of the fetal cells. The identification may be carried out in a laser scanner.

Example 2

Mounting on slides before fixation.

Pheripheral venous blood is obtained from the pregnant woman.

Slides are prepared by smearing the blood on poly-L-lysine coated slides, whereafter the slides are stored individually in plastic bags at –20 °C. The fixation is carried out as described in table III.

Table III

Step	Procedure: Fixation.	Time	Temperature
1	Fix cells in 2% paraformaldehyde in PBS.	10 min	RT
2	Permeabilize cells methanol/acetone (1:1)	10 min	-20 °C

Antibody staining and identification is as described in example 1. The cell morphology is maintained more consistently when mounting on slides before fixation is carried out.

Example 3

Verification of fetal cells by identifying the Y chromosomes

Fetal cells were diagnosed by the Y chromosome FISH analysis by the following procedure:

Maternal whole blood is smeared onto a slide, and fixed 2 minutes in paraformaldehyde (2 %) at room temperature. The cells are permeabilised 10 minutes in acetone at –20 °C, and then washed 3 minutes in PBS-buffer at room temperature. The slides are dehydrated in 62%, 96% and 99% alcohol at room temperature, and air-dried. A DNA probe 'pBAM-X' for the X-chromosome labelled with digoxigenin and a DNA probe 'ph-y 2,1' for the Y-chromosome labelled with biotin are used. The slide is placed on a heating plate at 37 °C, and the probe is added to the slide and a

cover glass placed on top of it and sealed with glue. The slide is placed immediately on a heating plate at 83,5 °C in 7 minutes, and hybridisation is conducted over night at 42 °C in moistened air.

After hybridisation the glue is removed and the slide is washed in 2xSSC until the cover glass releases, the in 0.4xSSC/0.3% NP-40 in 2 minutes at 73 °C. Then in 2xSSC/0.1% NP-40 in 1 minute at room temperature, and then rinsed in 2xSSC.

The slide is incubated 20 minutes at 37 °C with fluorescein-labelled avidin/rhodamin labelled anti-digoxigenin, and then washed 5 minutes in 4xSSC/0.1% tween 20, rinsed in 2xSSC and air dryed.

The slide is then mounted in Vectashield with counterstain added (DAPI or PI).

The identification of cells is as described in example 1.

The verification method may alternatively be a diagnostic method using probes relevant for the diseases and/or chromosome abnormalities to diagnose.

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Claims per July 16, 2001:

1. A method for isolating fetal cells from maternal blood, comprising

performing, on a sample of maternal blood having a size of from 0.5 to 40 ml, from which at the most 50 % of the maternal nucleated cells thereof have been removed, and/or at the most 50 % of the maternal anucleated cells have been removed,

selective labelling of fetal cells in the maternal blood sample,

identifying the selectively labelled fetal cells by scanning from 0.5 to 40 ml of the maternal blood sample, and

specifically isolating substantially only the selectively labelled fetal cells.

2. The method according to claim 1, wherein the selectively labelled cells are identified by scanning with a scanning rate of from 0.1 m/sec to 10 m/sec.

3. The method according to claim 1, wherein at the most 15 % of the maternal cells thereof have been removed.

4. The method according to claim 1, wherein at the most 10 % of the maternal cells thereof have been removed.

5. The method according to claim 1, wherein at the most 5 % of the maternal cells thereof have been removed.

6. The method according to claim 1, wherein at the most 2.5 % of the maternal cells thereof have been removed.

7. The method according to claim 1, wherein at the most 1% of the maternal cells thereof have been removed.

8. The method according to claim 1, wherein substantially none of the maternal cells have been removed from the sample.

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9. The method according to claim 1, wherein at the most 20 % of the maternal nucleated blood cells thereof have been removed.

10. The method according to claim 1, wherein at the most 20 % of the anucleated red blood cells thereof have been removed.

11. The method according to the claim 1, wherein substantially none of the anucleated blood cells have been removed from the sample.

12. The method according to claim 1, wherein at the most 20% of the anucleated red blood cells have been removed from the sample, and at the most 20% of the nucleated blood cells have been removed from the sample.

13. The method according to any of the preceding claims, wherein the maternal blood sample is diluted before labelling or identification of the fetal cells.

14. The method according to any of the preceding claims, wherein the selective labelling is based on hybridisation of a probe to m-RNA selectively expressed by fetal cells.

15. The method according to claim 14, wherein the m-RNA is m-RNA coding for a protein selected from the group consisting of embryonic hemoglobin, such as epsilon globin chains and zeta globin chains, and fetal hemoglobin, such as gamma globin chains, and alpha globin chains.

16. The method according to claim 14 or 15, wherein the hybridisation probe is directly labelled by having fluorochromes covalently attached thereto .

17. The method according to any of claims 1-13, wherein the selective labelling is based on an antigen-antibody reaction with a protein selectively produced by fetal cells.

18. The method according to claim 17, wherein the protein is a protein selected from the group consisting of embroynic hemoglobin, such as epsilon globin chains and

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zeta globin chains, and fetal hemoglobin, such as gamma globin chains and alpha globin chains.

19. The method according to claim 17 or 18, wherein the antibody is selected from anti epsilon (ε) antibodies, such as monoclonal unlabelled antibodies, and monoclonal fluorochrome labeled antibodies, anti zeta (ζ) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies, monoclonal fluorochrome labeled antibodies, and fluorochrome labeled antibodies, anti gamma (γ) antibodies, such as polyclonal (sheep) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies, monoclonal fluorochrome labeled antibodies, and fluorochrome labeled antibodies, anti alpha (α) antibodies, and anti beta (β) antibodies.

20. The method according to any of the preceding claims, wherein two or more selective labellings are performed to enhance the probability of identifying the fetal cells in the sample.

21. The method according to claim 20, wherein a labelling with a hybridisation probe is combined with a antigen-antibody labelling.

22. The method according to any of the preceding claims, wherein the identification of the selectively labelled fetal cells is taking place after spreading the blood sample on a solid surface and detecting labelled cells on the surface.

23. The method according to claim 22 wherein the position of detected labelled cells on the surface is recorded.

24. The method according to claim 23, wherein the detected cells the position of which has been recorded are collected.

25. A method of diagnosing a disease in a fetus comprising

obtaining a blood sample having a size of from 0.5 to 40 ml from the woman pregnant with said fetus, whereby at most 50 % of the maternal nucleated cells have

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been removed and/or at most 50 % of the maternal anucleated cells have been removed from said blood sample,

selective labelling the fetal cells in the maternal blood sample,

identifying the selectively labelled fetal cells, by scanning from 0.5 to 40 ml of the maternal blood sample,

specifically labelling with at least one disease marker the identified fetal cells for diseases, and identifying specifically labelled cells.

26. The method according to claim 25, wherein the fetal cells are isolated before specifically labelling the fetal cells with at least one disease marker.

27. The method according to claim 25, wherein the disease is a genetic disease and/or a chromosome abnormality.

28. The method according to claim 27, wherein the disease and/or chromosome abnormality is cystic fibrosis, hemophilia, muscular dystrophy, Down' syndrome, Klinefelter, Turner' syndrome.

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DECLARATION AND POWER OF ATTORNEY U.S.A.

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101 / 102

As a below named inventor, I declare that my residence, post office address and citizenship are stated below next to my name, the information given herein is true, that I believe that I am the origin first and sole inventor (if only one name is listed at 201 below), or an original, first and joint inventor (if plural inventors are named below at 201-203, or on additional sheets attached hereto) of the subje matter which is claimed and for which patent is sought on the invention entitled:

ISOLATION AND CULTURING OF FETAL CELLS

which is described and claimed in:

[X] PCT International Application No. **PCT/DK00/00281** filed **25 May 2000**

[ ] the attached specification

[ ] the specification in application Serial No. _____ filed _____

(if applicable) and amended on _____

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.
I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.
I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

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Prior Foreign Application(s) (Number)	(Country)	(Day/Month/Year Filed)	Priority Claimed Yes	No
99201651.9	European Patent Application	25 May 1999	[X]	[ ]
			[ ]	[ ]
			[ ]	[ ]

I hereby claim the benefit under Title 35, United States Code,§119(e) of any United States provisional application(s) listed below:

Application No. _____ Filing Date _____ Application No. _____ Filing Date _____

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I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status: patented, pending, abandoned)

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*Inventor(s) name must include at least one unabbreviated first or middle name.

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I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that thes statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under section 1001 of Title 18 of the Unite States Code; and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201*	SIGNATURE OF INVENTOR 202*	SIGNATURE OF INVENTOR 203*
X	X	
DATE X 3. January 2002	DATE X January 3, 2002	DATE

[ ] Additional inventors are named on separately numbered sheets attached hereto.